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APPLICATION NOTE 21848

NISTmAb characterization with a high-performance RP chromatography column

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MAbPac RP column, inter-column reproducibility, intra-column reproducibility, column lifetime, quantification, column and system carry-over, column recovery, wide linearity range, accurate and precise QCs, NISTmAb, Vanquish UHPLC, monoclonal antibodies, laboratory productivity

Application benefits

- Proven intra- and inter-column reproducibility in retention time with overall run-to-run %RSD at 0.12% and column-to-column %RSD at 0.33%
- Proven column robustness with minimal column degradation after more than 1000 monoclonal antibody injections
- Consistently low carry-over with up to 96.4% column recovery for accurate quantification
- Wide column calibration range from 0.1 to 20 µg for NISTmAb, with $R^2 > 0.999$ and average area count %RSDs < 2
- High accuracy and precision; all three different QC levels within 93–104% accuracy

Goal

To demonstrate the robustness, reproducibility, carry-over, recovery, accuracy, and precision of quantification of the NIST monoclonal antibody IgG1K (NISTmAb) using the Thermo Scientific™ Vanquish™ Horizon UHPLC integrated biocompatible system and Thermo Scientific™ MAbPac™ RP LC columns

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Introduction

The monoclonal antibody (mAb) therapeutics market is growing at a rapid rate owing to increased demand for targeted treatments. Therapeutic mAbs are usually produced from mammalian cells and can become heterogeneous due to post-translational modifications. Modifications such as oxidation can be introduced during the manufacturing process. Separation techniques to analyze mAb therapeutics in liquid chromatography (LC) include reversed-phase (RP), size exclusion (SEC), hydrophobic interaction (HIC) and ion exchange (IEX) modes. The MAbPac RP column is a RP column specifically designed for separation of intact mAbs and mAb fragments. The MAbPac RP column is based on 4 µm wide-pore polymer particles that are stable at extremes of pH (0–14) and temperature (up to 110 °C).[1] The wide pore size of polymeric particles enables efficient separation of protein molecules with low carry-over.

Accuracy, precision, robustness, and suitability are also of critical importance in analytical and biophysical methodologies. The NIST monoclonal antibody IgG1K (NISTmAb) is intended to provide a well-characterized, readily available reference material to facilitate analytical method development applications associated with the characterization of originator and follow-on biologics. NISTmAb is a recombinant humanized IgG1K expressed in murine suspension culture and is approximately 150 kDa. This homodimer consists of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds.[2,3]

This application note presents the benefits of separation of NISTmAb reference material 8671 (Lot No. 14HB-D-002) using the combination of the MAbPac RP column on a Vanquish Horizon UHPLC for optimum performance. Comparisons were made between the MAbPac RP polymer column and a standard 300 Å C4 silica column. Characteristics such as peak width at half height (PWHH), peak asymmetry, plate count (EP), and total peaks detected were measured.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ acetonitrile (ACN) (P/N A955-4)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), sequencing grade (P/N 28901)

Standards

NISTmAb, Humanized IgG1k Monoclonal Antibody, 10 mg/mL in 12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl (pH 6.0), was diluted to 1 mg/mL with water.

Equipment

Vanquish Horizon UHPLC system, including:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler HT (P/N VH-A10-A) with 25 µL sample loop
- Diode Array Detector HL (P/N VH-D10-A)

LC conditions

Columns:	MAbPac RP column, 2.1 × 50 mm, 4 µm, 1500 Å. polymeric based (P/N 088648) Thermo Scientific™ Biobasic™ C4 RP column, 2.1 × 50 mm C4, 5 µm, 300 Å silica column
Mobile phase A:	H_2O/TFA (99.9:0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90:9.9:0.1 v/v/v)
Flow rate:	0.6 mL/min
Column temp.:	80 °C
Sample volume:	1 µL
UV detector:	280 nm
Vial:	C5000-97
Mobile phase gradient:	Refer to Table 1

Table 1. LC gradient

Time (min)	%B
0	20
1	20
10	55
12	55
12.1	20
14	20

Chromatographic data processing and software

- Thermo Scientific™ Chromeleon™ CDS 7.2 SR5

Results and discussion

Chromatogram comparison: MAbPac RP column vs. silica-based wide-pore RP column

Figure 1 shows the UHPLC chromatograms of the NISTmAb with two different columns with the same separation conditions. Column A is a 5 µm silica-based RP protein column with pore size at 300 Å and column B is the MAbPac RP column, both were in 2.1 × 50 mm format. The MAbPac RP column showed a narrower peak shape and the detection of more peaks (Table 2). The zoomed in area illustrates that at least eight peaks were detected by the MAbPac RP column with one major lysine variant, while only one peak was shown with the silica-based RP column. Overall, this high-resolution supermacroporous polymer-based MAbPac RP column results in a superior separation of NISTmAb variants.

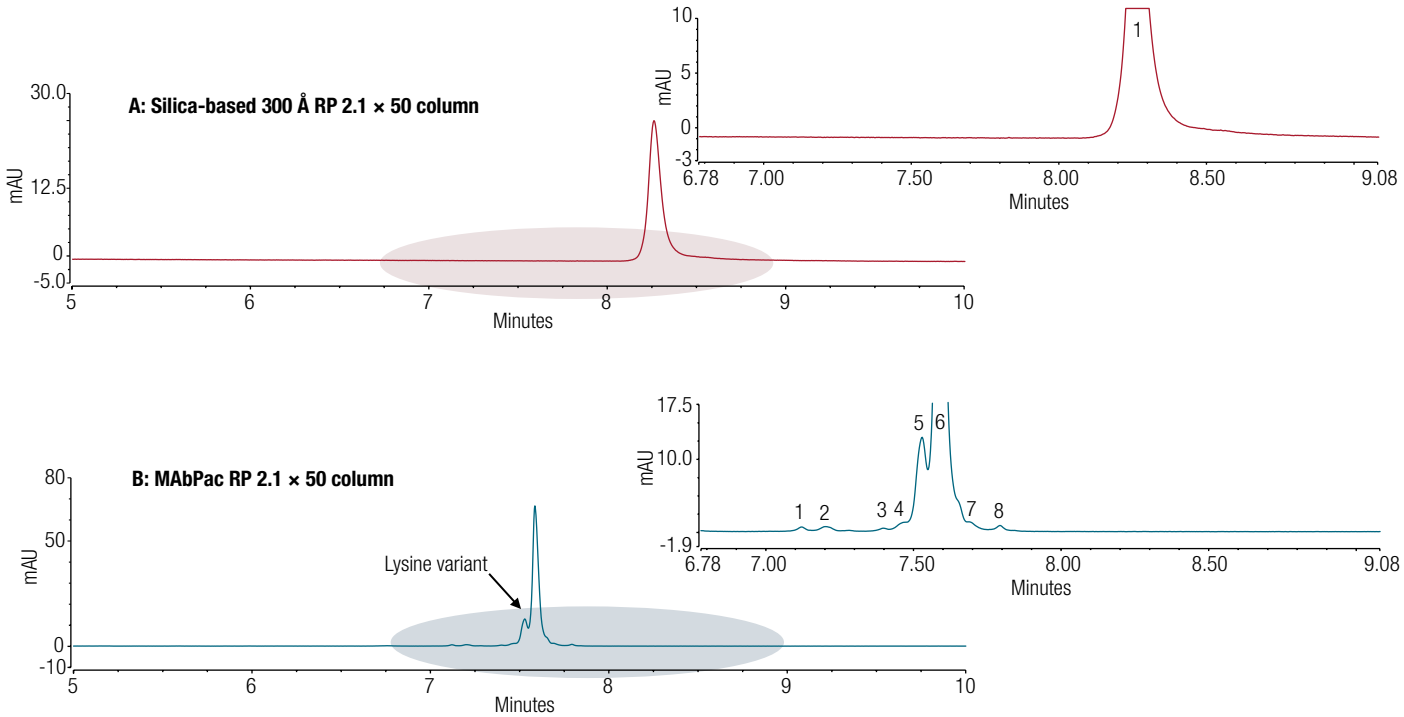


Figure 1. NISTmAb chromatogram: MAbPac RP column vs. silica-based wide-pore column

Table 2. Column performance

1 µg NISTmAb	Width (50%) (s)	Asymmetry EP	Total Peaks Detected
MAbPac RP column	2.2	0.95	8
Silica-based wide-pore column	4.4	1.38	1

Column lifetime – robustness test

Column lifetime is an important issue when developing an LC method. It can be affected by several factors including sample complexity, mobile phase composition, operating temperature, and storage. In mAb analysis, the separations usually require high temperatures to maintain peak shape of the protein and fragments. Operation at high temperatures (i.e., > 60 °C), can challenge the lifetime of conventional silica columns. Based on polymer technology, the MAbPac RP column can withstand temperatures up to 110 °C. The column lifetime was evaluated with 1100 continuous runs of 1 µg NISTmAb at 80 °C.

To ensure sample fidelity, NISTmAb samples were diluted with water, aliquoted, and stored at -20 °C. Each day of analysis, a fresh aliquot of NISTmAb sample was prepared for analysis. Figure 2 demonstrates that MAbPac RP column performance is maintained throughout 1100 runs at 80 °C. Consistent retention time, peak width at half height (PWHH), and peak shape with minimal increase in column backpressure is observed (Table 3). As demonstrated by more than 1000 runs with minimal retention time shift (0.12% RSD for NISTmAb and 0.13% RSD for its lysine variant), the MAbPac RP column is not affected by operation at high temperatures. Furthermore, the performance of the column is maintained over the course of the runs, with less than 1% RSD in NISTmAb EP peak asymmetry.

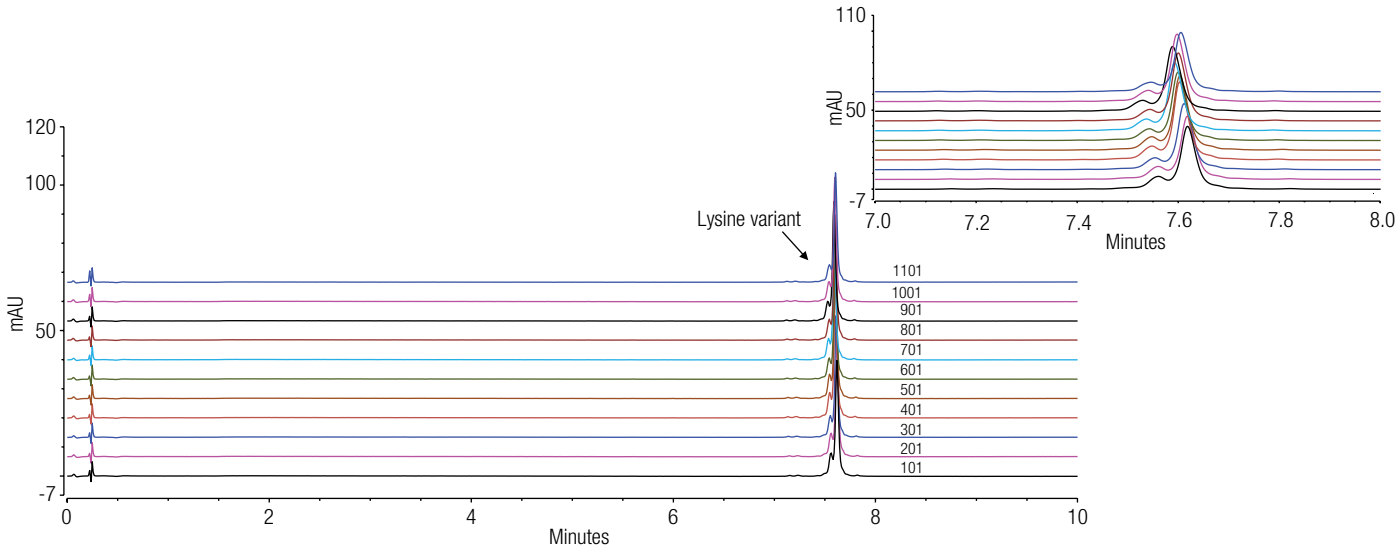


Figure 2. Column lifetime test - chromatogram overlay for >1000 injections

Table 3. %RSDs for RT, PWHH, and asymmetry for >1000 runs

1 µg NISTmAb	RT (s)	Width (50%) (s)	Asymmetry EP	RT (Lysine Variant)
RSD through 1100 runs	0.12%	2.17%	0.96%	0.13%

Column lot-to-lot reproducibility

Column reproducibility is an essential and critical requirement in chromatography to ensure smooth method transfer and good quality control. In this study, four random lots of MAbPac RP columns were evaluated with NISTmAb. MAbPac RP columns are manufactured using a high purity polymer and tightly controlled manufacturing processes. Consistent intra- and inter-column performance was achieved (Figures 2 and 3) with compelling chromatographic parameters. Overall lot-to-lot retention time reproducibility at 0.33% with NISTmAb and 0.30% RSD for its variant (Table 4) indicate superior column reproducibility with the MAbPac RP column.

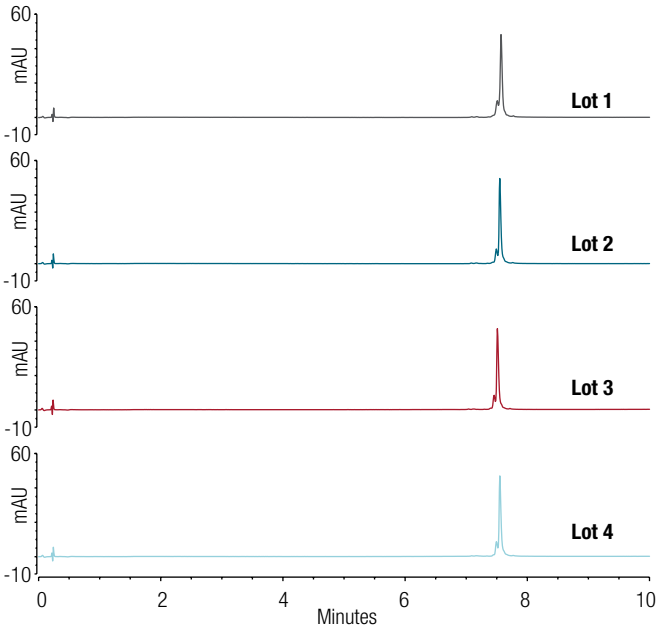


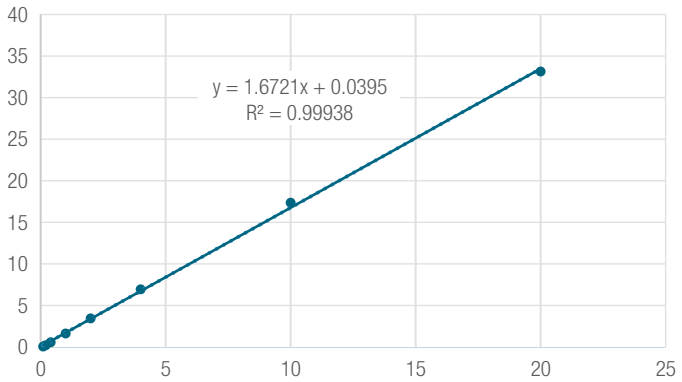
Figure 3. MAbPac RP 2.1 × 50 column lot-to-lot reproducibility (NISTmAb)

Table 4. Retention time lot-to-lot reproducibility

Lot #	RT	Area	Height	Width (50%)	Asymmetry	RT for Variant
1	7.570	2.4365	47.52	0.035	0.89	7.508
2	7.549	2.3275	49.43	0.032	0.95	7.492
3	7.511	2.2903	46.98	0.033	0.99	7.456
4	7.550	2.2493	46.67	0.033	1.00	7.493
RSD	**0.33%**	**3.46%**	**2.60%**	**3.78%**	**6.67%**	**0.30%**

Quantification results

Column loading capacity is an important consideration for laboratories screening samples from various sources. The amount of protein in the sample can vary widely. To show that the loading capacity of the MAbPac RP column can span three orders of magnitude, a calibration range of 0.1–20 µg was prepared. Linear response over the calibration range was observed, with R^2 values of 0.9994. Figure 4 shows the calibration curve of NISTMAb and the deviations for mass loading. Quality control samples were prepared at three concentrations (0.15, 0.7, and 7 µg) and all results were within 7% of expected values with average RSDs between 0.4% and 3.1% (Table 5).



Mass load (µg)	%RSD (n=3)
0.1	6.1
0.2	1.4
0.4	0.5
1	1.2
2	0.9
4	0.8
10	0.2
20	0.5

Figure 4. NISTmAb calibration curve R^2 = 0.9994; linearity range from 0.1 to 20 µg and all %RSD < 2 except the LLOQ at 6.1%

Table 5. Different QCs are within 90–110% in accuracy and < 4% in %RSD at n=4

Nominal	Measured	% Accuracy	%RDS (n=4)
0.15	0.14	93.3%	3.1
0.7	0.72	103.5%	0.4
7	6.74	96.2%	0.4

Column recovery

Loss of protein at low concentrations on HPLC columns is a common problem for silica-based columns. The usual cause is the presence of active sites on the packing surface that irreversibly bind proteins. Since the MAbPac RP column is based on high purity polymer chemistry, all silica-based interactions and metal contamination concerns are alleviated. Figure 5 demonstrates 1 µg injected on the column with 96.4% recovery for the MAbPac RP column while with a silica-based wide-pore column only 87% recovery was obtained.

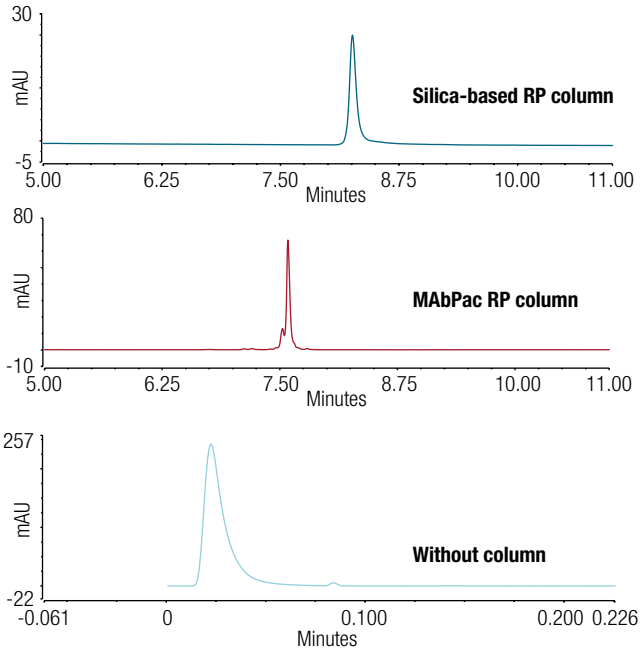


Figure 5. NISTMAb recovery on the silica-based RP column and the MAbPac RP column

Carry-over

Within the field of quantification, one of the biggest limitations of an analytical procedure is the dynamic range of the assay. Carry-over or memory effect can have consequential effects in many areas where separation science is used, from the world of bioanalysis to that of forensics to that of environmental studies.[4] The issue that arises is that unless the entire sample is removed from the analytical system, the subsequent analysis will have residual compound from the previous injection, which could potentially lead to inaccurate data being produced.

There are several components that can act as source of carry-over in a chromatographic system including the column, detector, autosampler, tubing, contaminated blank samples, and mobile phase. In this assay, column carry-over is considered separately from overall system carry-over. The focus is to understand how much NISTmAb, a relatively hydrophobic mAb, is irreversibly retained by the column. To determine this column-related carry-over a second gradient is run immediately following the sample analysis, without making another injection.[5] In Figure 6, the zoomed in area shows the column carry-over, with 1 µg NISTmAb mass loading calculated as 0.16%. The procedure was used for mass loading ranged from 0.02-20 µg, shown in Table 6 and all levels tested showed < 0.3% of the loading amount. The ability to quantitate proteins from 0.1 to 20 µg with minimal carry-over makes this an appropriate method for accurate and consistent quantitation.

Table 6. System and column carry-over with different NISTmAb mass loading

Mass load (µg)	Column carry-over (%)
0.02	NA
0.04	NA
0.1	NA
0.2	NA
0.4	0.08%
1	0.16%
2	0.19%
4	0.22%
10	0.23%
20	0.25%

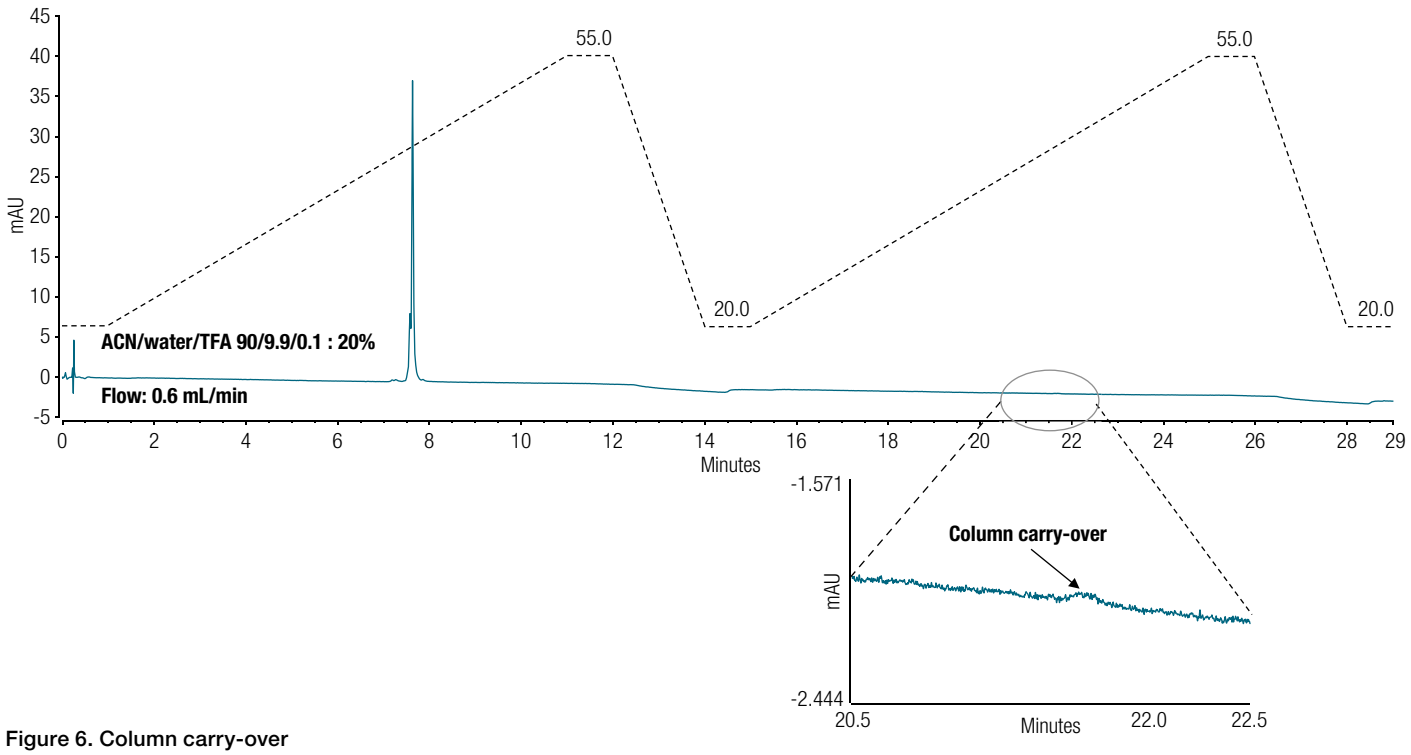


Figure 6. Column carry-over

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Conclusion

The NISTmAb reference material, RM 8671, is intended for use in evaluating the performance of methods for determining physicochemical and biophysical attributes of monoclonal antibodies. It also provides a representative test molecule for development of novel technology for therapeutic protein characterization.[3] The MAbPac RP column coupled to a Vanquish UHPLC system formed a robust platform and an excellent choice for mAb characterization. Excellent peak shape and column efficiency was demonstrated. Additionally, the superior resolving power, lot-to-lot column reproducibility, and column lifetime demonstrate the consistency and robustness of the column. Finally, minimal column carry-over and high recovery results in the MAbPac RP column being an excellent choice for applications requiring a wide dynamic range and accurate and precise quantification of QCs.

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